Stratification of asthma phenotypes by airway proteomic signatures

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on behalf of the U-BIOPRED Study Group

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Background: Stratification by eosinophil and neutrophil counts increases our understanding of asthma and helps target therapy, but there is room for improvement in our accuracy in prediction of treatment responses and a need for better understanding of the underlying mechanisms.

Objective: We sought to identify molecular subphenotypes of asthma defined by proteomic signatures for improved stratification.

Methods: Unbiased label-free quantitative mass spectrometry and topological data analysis were used to analyze the proteomes of sputum supernatants from 246 participants (206 asthmatic patients) as a novel means of asthma stratification. Microarray analysis of sputum cells provided transcriptomics data additionally to inform on underlying mechanisms.

Results: Analysis of the sputum proteome resulted in 10 clusters (ie, proteotypes) based on similarity in proteomic features, representing discrete molecular subphenotypes of asthma. Overlaying granulocyte counts onto the 10 clusters as metadata further defined 3 of these as highly eosinophilic, 3 as highly neutrophilic, and 2 as highly atopic with relatively low granulocytic inflammation. For each of these 3 phenotypes, logistic regression analysis identified candidate protein biomarkers, and matched transcriptomic data pointed to differentially activated underlying mechanisms.

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Conclusion: This study provides further stratification of asthma currently classified based on quantification of granulocytic inflammation and provided additional insight into their underlying mechanisms, which could become targets for novel therapies. (J Allergy Clin Immunol 2019;144:70-82.)

Key words: *Asthma, proteomics, biomarkers, eosinophils, neutrophils*

Asthma is a heterogenous disease involving inflammatory and structural cells and a multitude of molecular mediators. Development of new drugs, including biologics,[1] with stratified medicine will improve patient outcomes. Current phenotypes of asthma are defined predominantly by clinical characteristics and a limited number of biomarkers.[2] The most widely studied asthma biomarkers to date are sputum and blood eosinophils, exhaled breath nitric oxide (eNO), and serum periostin.[3] Blood eosinophilia predicts the risk of acute asthma exacerbations,[4] and treatment strategies incorporating normalization of sputum eosinophil counts have resulted in marked reductions in exacerbations.[5] Eosinophilia has been used to enrich target populations in trials of the anti–IL-5 mAbs. By comparison, defining asthma phenotypes by using other inflammatory cell types has been less rewarding.

In the first project to apply multiple omics methodologies in an unbiased manner to stratify asthma, Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED) has shed light on conceptually novel mechanisms and phenotypes. In the current study (see Fig E1 in this article's Online Repository at www.jacionline.org), quantitative data-independent liquid chromatography with high-definition mass spectrometry was used to profile proteomes of the lining fluid of the bronchial epithelium sampled by means of sputum induction. We hypothesized that clustering of sputum proteomic data would identify molecular phenotypes at higher resolution than the existing asthma eosinophilic, neutrophilic, and paucigranulocytic phenotypes based on sputum granulocyte counts. By investigating molecular pathways, defined by using a whole-genome array from sputum cells of the same participants, we have begun to understand the mechanisms that underlie these new phenotypes.

METHODS

Study design

U-BIOPRED is a multicenter study in which participants were phenotyped by using standardized protocols, lung function testing, and assessment of atopy[6] and applying several omics-unbiased technologies (genomics, transcriptomics, proteomics, lipidomics, breathomics, and metabolomics) to enable novel concepts of asthma mechanisms and phenotypes to be developed. In this study we report the results of proteomic analysis of sputum supernatants as a means of studying the bronchial epithelial lining fluid sampled by means of standard sputum induction, which is widely used in asthma research. Of the total 610 adult participants enrolled into U-BIOPRED, 246 provided sputum samples that passed stringent quality control for proteomic analysis: 118 nonsmoking patients with severe asthma, 48 current or ex-smoking patients with severe asthma, 40 patients with mild-to-moderate asthma, and 40 healthy participants (Table I).

Abbreviations used

ACQ:	Asthma Control Questionnaire
eNO:	Exhaled breath nitric oxide
ERAD:	Endoplasmic reticulum–associated degradation
Hrd1:	Synoviolin 1
IPA:	Ingenuity Pathway Analysis
IRE1α:	Inositol-requiring enzyme 1 α
NF-κB:	Nuclear factor κB
OCS:	Oral corticosteroid
ROC:	Receiver operating characteristic
TDA:	Topological data analysis
U-BIOPRED:	Unbiased Biomarkers for the Prediction of respiratory disease outcomes

Sputum induction and proteomic analysis

Sputum collection and analysis were performed according to methods detailed by Burg et al.[7] Proteins were extracted from sputum supernatants by means of precipitation and analyzed in duplicates through liquid chromatography with mass spectrometry using a Waters G2Si mass spectrometer coupled to a nanoAcquity UPLC (Waters, Milford, Mass). Patient-matched cell pellets processed into RNAlater were assessed for global gene expression by using Affymetrix HT HGU133+ microarray analysis. For a full set of microarray data, see the previous report.[8]

Data analysis

Patients were randomized into a training and test set with a 3:7 ratio. All proteomic data were $\log_2$ transformed. Batch effects were corrected by using ComBat.[9] Statistical analyses were performed in R software, custom Python scripts, and Microsoft Excel. Clustering of patients was based solely on proteomic data using topological data analysis (TDA) performed in Ayasdi Core software (Ayasdi, Menlo Park, Calif), applying a norm correlation metric and 2 MDS lenses (resolution 32, gain 3.4×, equalized). Subphenotypes of asthma were assigned based on the persistence of TDA structure and conserved groupings of nodes (1) when changing sampling settings and modifying input data (eg, log vs natural data), (2) when varying the cohort input (eg, all participants vs asthmatic participants only), and (3) through use of metadata from consensus models and experimental composition (see this article's Online Repository at www.jacionline.org). Cluster boundaries were created through an iterative process of varying resolution and gain settings and comparison of the different analytic data sets. For comparison and validation of the TDA approach, the same data were also clustered by using the consensus cluster plus R package, applying partitioning around medoids (PAM) and Pearson correlation with settings of a maximum k value 20 and 1000 repetitions with item resampling setting of 0.9 (see Fig E2 in this article's Online Repository at www.jacionline.org).

To identify individual predictive proteins that could be used in future studies as candidate biomarkers, we applied elastic net–regularized logistic regression to data in the training set and then tested the predictive value of the proteins by using the receiver operating characteristic (ROC) curve in the test set. Where it was not appropriate to break up data into training and test data sets because of sample size, exploratory analysis to identify predictive biomarkers was performed with elastic net–regularized logistic regression alone applied to the combined training and test data set, comparing the cluster of interest with the rest of the participants. Logistic regression and ROC curves were conducted by using scikit-learn in Python.

Pathway analysis of patient-matched sputum transcriptomics data

RNA extracts from sputum cell pellets from the same samples from which sputum supernatants were analyzed for protein content by using high-definition mass spectrometry were subjected to microarray analysis and Ingenuity Pathway Analysis (IPA).

TABLE I. Participant demographics

	Severe nonsmoking asthmatic patients	Smokers and ex-smokers with severe asthma	Mild-to-moderate nonsmoking asthmatic patients	Healthy nonsmoking control subjects
Subjects (no.)	117	45	38	40
Age (y)	52.88 ± 13.07	55.2 ± 10.25	42.37 ± 15.23	37.2 ± 13.11
Age at diagnosis (y)	20.32 ± 16.42	33.53 ± 19.87	16.23 ± 16.85	NA
Sex ratio (male/female)	43/74	18/27	21/17	28/12
BMI ($kg \cdot m^{-2)}$	28.98 ± 6.61	30.1 ± 6.47	25.44 ± 4.61	25.61 ± 3.19
BMI >30 $kg \cdot m^{-2}$	46	21	9	4
Serum IgE ($IU \cdot mL^{-1}$)	274.88 ± 440.53	385.09 ± 1046.47	326.32 ± 660.16	87.66 ± 140.24
FEV_1 (% predicted)	65.44 ± 21.68	65.33 ± 17.2	90.68 ± 18.48	101.89 ± 12.72
FCV (% predicted)	87.67 ± 19.89	89.67 ± 16.8	107.91 ± 18.15	108.71 ± 12.91
FEV_1/FVC ratio	60.11 ± 13.45	59.35 ± 11.1	70.06 ± 10.87	NA
Exacerbations in previous year	2.27 ± 1.79	2.6 ± 2.46	0.45 ± 0.93	NA
Smoking history (pack-years)	2.46 (0-5)	23.7 (5-70)	3.62 (1-5)	2.25 (0-5)
Intubation (ever)	13	1	0	NA
ICU admission (ever)	28	7	0	NA
Positive atopy test result	83	26	39	14
OCS	48 (40.6%)	24 (50%)	0 (0%)	
Prednisolone (equivalent [mg])	11.81 ± 6.94	13.75 ± 8.95	0 ± 0	
Inhaled corticosteroids	114 (96.6%)	44 (97.9%)	38 (100%)	
Long-acting β-agonist	113 (95.7%)	45 (93.7%)	1 (2.5%)	
Short-acting β-agonist	92 (77.9%)	35 (72.9%)	30 (75%)	
Injected corticosteroids*	8 (6.7%)	0 (0%)	0 (0%)	
Long-acting muscarinic antagonist	30 (25.4%)	13 (27%)	0 (0%)	
Short-acting muscarinic antagonist	51 (43.2%)	22 (45.8%)	0 (0%)	
ACQ				
Mean ACQ5	2.21 ± 1.18	2.13 ± 1.13	0.81 ± 0.68	
Mean ACQ7	2.56 ± 1.25	2.55 ± 1.04	0.95 ± 0.65	
AQLQ				
Total	4.55 ± 1.22	4.54 ± 1.14	5.86 ± 1.05	
Symptoms	4.51 ± 1.31	4.53 ± 1.21	5.88 ± 1.1	
Emotional	4.65 ± 1.61	4.7 ± 1.52	6 ± 1.21	
Environmental stimuli	4.83 ± 1.45	4.55 ± 1.45	5.59 ± 1.36	
Activity limitation	4.45 ± 1.26	4.48 ± 1.22	5.86 ± 1	

Data are presented as means ± SDs, unless otherwise stated.
AQLQ, Asthma Quality of Life Questionnaire; *BMI*, body mass index; *FVC*, forced vital capacity; *ICU*, intensive care unit; *NA*, not applicable.
*Hydrocortisone and triamcinolone doses were converted to equivalent prednisolone dose.

RESULTS

The main demographic characteristics and medications of participants from the U-BIOPRED cohort whose data were used in this study (n = 246) are shown in Table I.

Stratification of asthmatic patients by protein signatures

By using our previously reported rationale for selecting sets of proteins for analysis,[7] 270 proteins identified and quantified in 40% or more of samples constituted the core data set for statistical analysis (see Table E1 in this article's Online Repository at www.jacionline.org). Within the network constructed using TDA, 10 clusters were identified, representing phenotypes of asthmatic patients with distinct proteomic signatures that we termed proteotypes (Fig 1). When sputum granulocyte counts were overlaid as metadata onto the TDA network, a strong association with sputum eosinophil and neutrophil counts across proteotypes was observed: proteotypes 1, 2, and 3 were highly eosinophilic (mean counts, 18.7%, 23.1%, and 17.0%, respectively) and were therefore defined as highly eosinophilic subphenotypes 1, 2, and 3. Collectively, they represented a common and highly eosinophilic phenotype comprising 31% of the cohort, with a mean eosinophil count of 21.1%. Clusters (subphenotypes) 5 and 6 had atopy as their main shared feature. Clusters (subphenotypes) 8, 9, and 10 were characterized by increased neutrophil counts (71.8% for all 3 combined [ie, the neutrophil phenotype], $P = 1.03E^{-9}$ when compared with the rest of the participants), with counts progressively increasing from subphenotypes 8 to 10 (mean, 63.5%, 82.0%, and 72.6% for proteotypes 8, 9, and 10, respectively). Two smaller and less well-defined phenotypes (composed of single proteotypes) were also identified: a mildly eosinophilic proteotype 4 (mean eosinophil count, 7.7%) and a mildly neutrophilic proteotype 7 (mean neutrophil count, 46.2%).

Subphenotypes of eosinophilic asthma

Sputum eosinophil counts in proteotypes 1, 2, and 3 were significantly ($P = 5.49E^{-9}$) greater than the rest of the TDA network (mean, 5.4%; Fig 2 and Table II), with 80 sputum proteins differentially abundant compared with healthy conditions ($P < .05$, see Table E2 in this article's Online Repository at www.jacionline.org) and 49 proteins compared with the rest of the asthmatic patients ($P < .05$, see Table E3 in this article's Online Repository at www.jacionline.org). Additionally, 14 proteins were differentially abundant at a significance P value of less than .05 when comparing individual eosinophilic

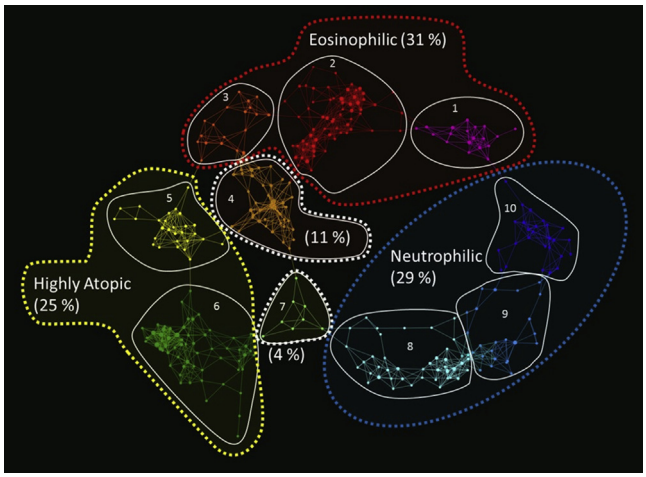

FIG 1. Asthma sputum proteomes/proteotypes/subphenotypes. The data network was created by using TDA of protein data sets from all asthmatic (mild/moderate and severe) participants and consisted of 10 clusters that we termed proteotypes. Differently colored nodes and edges denote different clusters. *Dotted boundaries* represent clusters grouped according to shared granulocyte and atopy profiles, whereas proteome clusters delineated by continuous boundaries represent subphenotypes. Connections *(lines)* between nodes represent overlap of patients between nodes/clusters. The percentage of participants in each phenotype is displayed adjacent to the phenotype name.

subphenotypes (Table II). Unlike the subphenotypes, the 3 major phenotypes were represented by sufficient patient numbers in the predefined, randomly assigned, training, and test cohorts to allow testing of the predictive success of associated proteins (training/test ratios were 40/22, 29/21, and 38/21 for highly eosinophilic, highly atopic, and highly neutrophilic phenotypes, respectively). Logistic regression/ROC analysis showed that 10 proteins were strongly predictive of the entire eosinophilic phenotype (Fig 3 and see Fig E3, *A*, in this article's Online Repository at www.jacionline.org): histone H4, vitronectin, histidine-rich glycoprotein, immunoglobulin heavy constant γ3, complement C3, transthyretin, serotransferrin, and α-1-antitrypsin (all increased relative to the rest of the cohort) and galectin-3–binding protein and ezrin (both reduced).

Levels of other biomarkers from the U-BIOPRED database were also increased in this phenotype, including blood periostin (Fig 2, *D*), a biomarker strongly associated with the T_H2 cytokine phenotype; eosinophilia; and airway remodeling (Fig 3, *A*, and Table II)[4] and sputum IL-13 (Fig 2, *B* and *C*).

Slightly fewer than a third of patients with this phenotype were receiving oral corticosteroids (OCSs), and 55% were atopic, with more frequent respiratory tract infections (93.5% vs 67.0%, $P = 8.7E^{-7}$), use of long-acting β-agonists (83.9% vs 58.5%, $P = 1.5E^{-4}$), and higher eNO concentrations (44.4 ± 35.8 ppb; Table II) implying more severe asthma than the rest of the cohort. Subphenotype 1 asthmatic patients had a greater prevalence of sinusitis (69.2% vs 30.6% of participants in subphenotypes 2 and 3). Eosinophilic subphenotype 2 had the highest eNO levels (mean ± SD, 48.77 ± 36.83 ppb) and the highest sputum eosinophil counts (23.08%), whereas subphenotype 3 had an increased frequency of atopy (81.8% compared with 37.3% in the other eosinophilic subphenotypes).

Subphenotypes of neutrophilic asthma

The neutrophilic phenotype, which is composed of subphenotypes 8, 9, and 10 (Fig 2 and Table II), represented 29.5% of the asthmatic patients and had significantly greater sputum neutrophil counts ($P = 1.03E^{-9}$). By comparison with other asthmatic patients, these patients had 134 differentially abundant proteins (see Tables E4 and E5 in this article's Online Repository at www.jacionline.org). Fourteen proteins were differentially abundant between the neutrophilic subphenotypes (Table III). Within this region of the TDA network, there was a concentration gradient of neutrophil-derived proteins, increasing from the left to the right side of the network (Fig 2, *J* and *K*): S100s, plastin-2, leukocyte elastase inhibitor, matrix metalloproteinase 9, and leukotriene A_4 hydrolase. This was associated with greater concentrations of sputum S100-A9 and matrix metalloproteinase 9 proteins. The 10 most predictive proteins identified by means of logistic regression/ROC analysis for this phenotype were histone H4 (also predictive of the eosinophilic phenotype), azurocidin, coronin-1A, chloride intracellular channel protein 1, annexin A1 and A3, neutrophil gelatinase-associated lipocalin (all increased in this phenotype), and transthyretin (reduced; Fig 3 and see Fig E3, *A*).

The number of intensive care unit admissions in the last 12 months was greatest in the neutrophilic phenotype (Fig 2, *L*), and their asthma symptoms were more likely to be triggered by fungus (54.2% vs 23.6%, $P = 1.4 \times 10^{-5}$) and dust (81.4% vs 51.3%, $P = 2.3E^{-5}$). OCS consumption was greatest in neutrophilic subphenotypes 8 to 10, lowest in the highly atopic subphenotypes 5 and 6, and intermediate but heterogeneous in eosinophilic subphenotypes 1 to 3 (Fig 2 and Table II). However, statistical analysis showed that OCS use was significantly greater (compared with other asthmatic patients) only in subphenotype 9, which was also associated with decreased β-agonist reversibility. When compared with neutrophilic subphenotypes 8 and 9, subphenotype 10 (5.5% of asthmatic patients) was associated with greater frequency of intensive care unit admissions (63.6% compared with 20.8% in subphenotypes 8 and 9, $P = 8.9E^{-3}$) but less rescue inhaler use ($P = 3.9E^{-3}$).

Subphenotypes of highly atopic asthma

This group constituted 25% of all asthmatic patients (Fig 2, *E*) characterized by mild asthma with the highest quality of life (Asthma Control Questionnaire [ACQ] score lower and Asthma Quality of Life Questionnaire score higher) and overall good lung function (mean FEV_1, 78% of predicted value) but a higher prevalence of atopy (78% vs 53% in the other asthmatic patients, $P = 9 \times 10^{-4}$; Table II) and total serum IgE concentrations (Fig 2, *F* and *H*). They had high levels of sputum uteroglobin and clusterin proteins (Fig 2, *E* and *G*). By comparison with the other asthmatic patients and healthy participants, 134 and 20 proteins, respectively, were differentially abundant (see Tables E6 and E7 in this article's Online Repository at www.jacionline.org). The predictive biomarkers for this phenotype are also shown in Fig 3 and Fig E4, *A*, in this article's Online Repository at www.jacionline.org.

Comparisons of the 2 highly atopic subphenotypes showed significantly ($P = .005$) greater abundance of lysozyme C in subphenotype 6 when compared with subphenotype 5. Conversely, eNO levels were significantly ($P = .002$) lower in subphenotype 6, with a trend toward lower ACQ and higher Asthma Quality

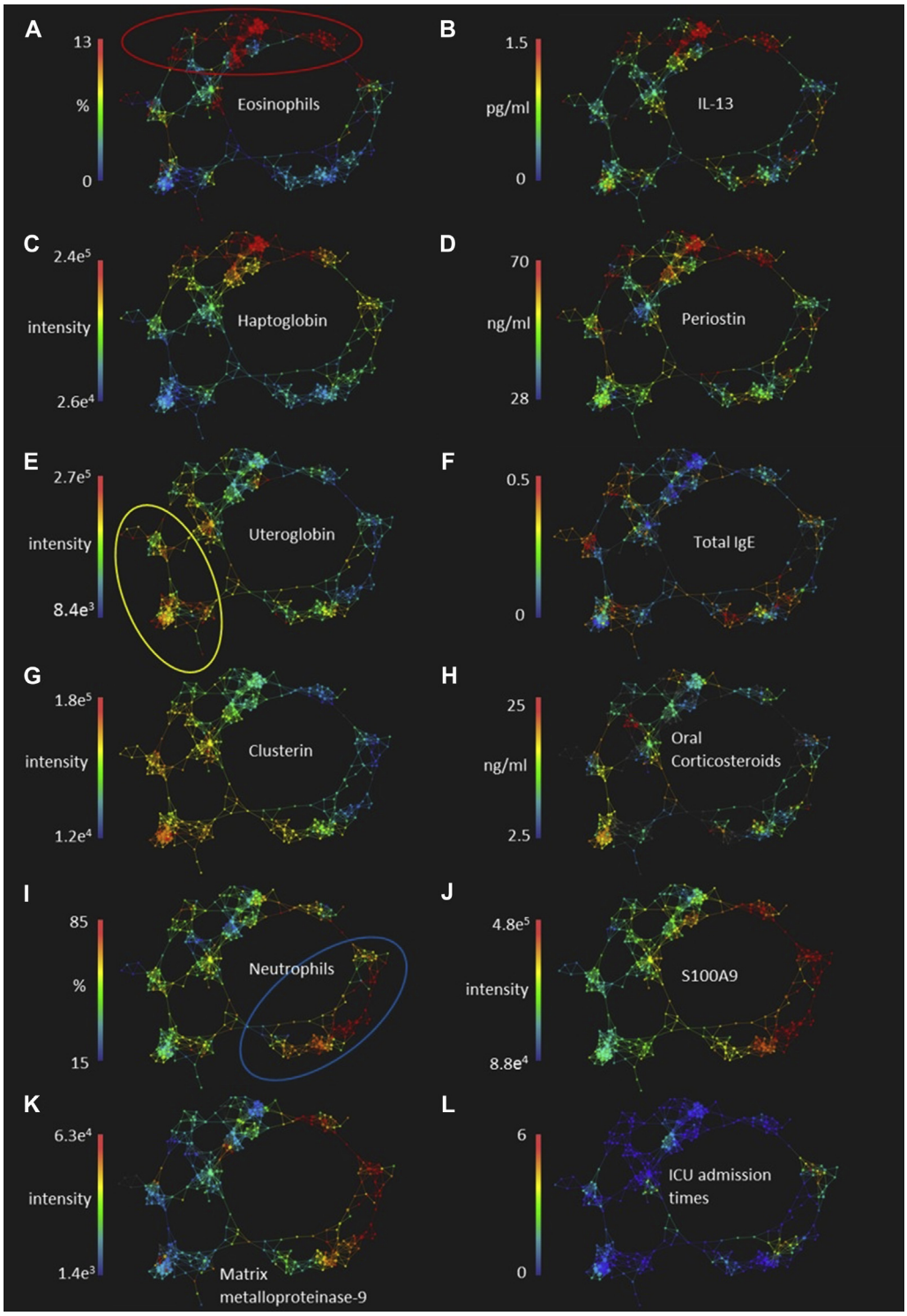

FIG 2. Pathobiological, clinical, and protein features associated with proteomes identified in asthmatic patients. **A-D,** Patients of an eosinophilic phenotype patients (circled red in Fig 2, *A*) had increased blood periostin (Fig 2, *D*), IL-13 protein (Fig 2, *B*), and sputum haptoglobin (Fig 2, *C*) levels. **E-H,** Patient of the highly atopic phenotype (circled in yellow in Fig 2, *E*) had high levels of sputum uteroglobin and clusterin proteins (Fig 2, *E* and *G*) and high total IgE levels and OCS doses (Fig 2, *F* and *H*). **I-K,** Patients of the neutrophilic subphenotypes (circled blue in Fig 2, *I*) had greater concentrations of sputum S100-A9 and matrix metalloproteinase 9 *(MMP9)* proteins (Fig 2, *J* and *K*). **L,** The number of intensive care unit admissions in the last 12 months was greatest in the neutrophilic phenotype. Colors denote concentrations of individual variables, ranging from blue (low) to red (high); see the *vertical intensity bar* alongside each panel.

of Life Questionnaire scores in subphenotype 5, suggesting that subphenotype 5 (9% of U-BIOPRED asthmatic patients) was less severe. Of note, subphenotype 5 had the greatest incidence of atopy among all asthma subphenotypes and a greater frequency of active hay fever but also had the greatest FEV_1 and forced vital capacity of all subphenotypes. Lysozyme C was more abundant in subphenotype 6 compared with subphenotype 5 (Table III), possibly because of greater neutrophil cell counts (Table II).

Pathway analysis of patient-matched sputum transcriptomics data

Subphenotypes were next compared for upstream regulators by applying IPA to the transcriptomics data set. This revealed a general trend (ie, a broad pattern) when moving from left to right across the TDA structure, with decreasing T2/atopy-associated and increasing T1 gene expression (Fig 4) when placing the clusters approximately in the order shown in Fig E6 and Table E9 in this article's Online Repository at www.jacionline.org. The same analysis showed that gene expression in the neutrophilic subphenotypes was predicted to result from downregulation of the T2 cytokine IL-13. Expression of miRNA for the T2 cytokine IL-5 was greatest in the eosinophilic subphenotypes (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). The pleiotropic cytokine thymic stromal lymphopoietin was detected at low abundance and was not clearly distributed between the subphenotypes; however, some of the greatest thymic stromal lymphopoietin expression was measured in eosinophilic subphenotypes 1 and 2.

The TDA network was also characterized by increasing activation of IL-2 from left to right. Neutrophilic subphenotypes had gene expression profiles predicted to result from downregulation of *COL18A1*, a gene associated with atopy. Additionally, there was a left to-right trend of predicted activation of virally induced transcription factors: IFN-α, KDM5B, and TNF. The top canonical pathways and upstream regulators of gene expression were predicted by using IPA analysis, and the top upstream regulator for each subphenotype is shown in Table E8 in this article's Online Repository at www.jacionline.org.

DISCUSSION

Using unbiased proteomic profiling of induced sputum supernatants, we have achieved a greater degree of stratification than currently possible by granulocyte count alone, substratifying eosinophilic and neutrophilic phenotypes each into 3 subphenotypes. Application of TDA provided a new perspective on stratification by creating a network with patient clusters defined by shared airway proteomes. Asthma severity increased across the network, with the most severe forms being at the extreme right end, where neutrophilia was a striking feature. Analysis of sputum cell transcriptomes from the same patients pointed to mechanistic pathways that could inform further optimization of asthma biologics and help in development of new asthma drugs. Logistic regression and ROC analysis identified several candidate biomarkers to be explored further for application in clinical practice, possibly when selecting patients for novel drugs and existing biologic treatments.

As anticipated, the eosinophilic phenotype showed patterns of sputum cell gene expression normally associated with eosinophilia (ie, IL-4 and IL-13; see Fig E6) and greater levels of serum IL-13 (Fig 2, *B*), epithelium-derived IL-13–induced biomarkers (see Fig E6, *C*), periostin (Fig 2, *D*), and exhaled nitric oxide (Table II). Of note, only 50% of these patients were atopic, less than the neutrophilic phenotype (68%, Table II), which is in keeping with the notion that T2 mechanisms are found in both atopic and nonatopic asthmatic patients. However, there were other, as yet unrecognized associations.

Differentially expressed genes were predicted to result from downregulated synoviolin 1 (Hrd1)–mediated signaling (see Table E8), a pathway that might be partially responsible for eosinophilia. Hrd1 mediates clearance of misfolded proteins through endoplasmic reticulum–associated degradation (ERAD),[10] and epithelial inflammation results from inhibition of Hrd1-associated ERAD.[11] Under normal conditions, the Hrd1-ERAD complex degrades endoribonuclease inositol-requiring enzyme 1 α (IRE1α; a sensor of unfolded protein response); in response to endoplasmic reticulum stress, this degradation is inhibited, and active IRE1α accumulates, causing epithelial inflammation through inflammatory mediators, such as c-Jun N-terminal kinase. Active IRE1α causes increased expression of Xbp1, which is highly activated during eosinophil commitment from granulocyte-monocyte progenitors.[12] Additionally, gene expression signatures of eosinophilic subphenotypes 1, 2, and 3 were predicted to result from activation of the nuclear retinoic acid receptor α.

Consistent with the knowledge of risk factors for asthma exacerbations,[13] the eosinophilic phenotype was associated with a high prevalence of reported respiratory tract infections, use of long-acting β-agonists, and gastro-oesophageal reflux disease. These patients had worse lung function and poor asthma symptom control, and 8% had been admitted to intensive care units because of exacerbations.

Ten proteins were strongly predictive of the eosinophilic phenotype. α-1-Antitrypsin neutralizes neutrophil elastase, which is known for its role in lung damage, bronchoconstriction, and airway hyperreactivity, and therefore its higher levels suggest negative feedback but also possibly interference with neutrophil-mediated anti-infection mechanisms. Serotransferrin is involved in transporting vitamin A (retinol)–bound retinol-binding protein and sequesters iron from invading bacteria, thereby inhibiting their ability to replicate and cause disease.[14] It has been shown that amyloid fibrils from 3 different sources (α-synuclein, Sup35, and transthyretin) induce NADPH oxidase–dependent neutrophil extracellular traps *in vitro* from human neutrophils.[15] Transthyretin plasma levels have been reported to decrease during systemic inflammation.[16] We and others have previously observed reduced sputum transthyretin levels in patients with chronic obstructive pulmonary disease, a neutrophilic airways disease, and now show that in asthmatic patients the levels are increased in eosinophilic inflammation. Higher levels observed in asthmatic patients in the current study might also reflect corticosteroid use, as suggested previously in an animal model.[16]

The neutrophilic phenotype was observed in about a third of asthmatic patients. Their symptoms were more likely triggered by dust or fungus, which is consistent with their high prevalence of atopy. Lower levels of transthyretin observed in this phenotype, which was among the set of 10 predictive proteins, is surprising considering the greatest percentage of OCS use in the highly neutrophilic phenotype (Table II). Other predictive proteins (Fig 3 and see Fig E4, *E*) included the neutrophil granular proteins, neutrophil gelatinase-associated lipocalin, azurocidin, S100A9,

TABLE II. Clinical features associated with eosinophilic and neutrophilic asthma subphenotypes

Asthma phenotype	Eosinophilic					Highly atopic
Asthma subphenotype	1	2	3	1, 2, 3	4	5
No. of participants	13	38	11	**62**	22	18
Percentage of asthmatic patients	6.5%	19.0%	5.5%	**31.00%**	11.0%	9.0%
Age (y)	52.46 ±13.43	52.57 ±13.15	**59.36 ±10.84**	53.75 ±12.91	51.31 ±11.4	48.5 ±16.34
Smoking (pack-years)	3.35 ± 8.52	6.72 ± 15.87	2.88 ± 4.75	5.33 ± 13.19	9.68 ± 17.59	*1.78 ± 4.52*
Mean ACQ5	2.16 ± 1.34	1.82 ± 1.4	1.96 ± 1.66	1.91 ± 1.42	1.64 ± 1.09	*1.16 ± 0.96*
Mean ACQ7	2.30 ± 1.35	2.07 ± 1.53	2.25 ± 1.81	2.15 ± 1.53	2.05 ± 1.19	*1.35 ± 1.08*
Mean AQLQ	4.76 ± 1.26	4.3 ± 2.09	*3.08 ± 2.29*	4.18 ± 2.03	4.78 ± 1.62	**4.99 ± 1.58**
Admitted to ICU	7.69%	10.53%	*0.00%*	8.06%	9.09%	22.22%
Oral steroids	30.76%	28.95%	27.27%	29.03%	36.36%	*22.22%*
Blood periostin (ng/mL)	**53.97 ± 31.72**	52.73 ± 26.63	50.52 ± 33.14	52.6 ± 28.45	35.88 ± 20.27	41.72 ± 33.65
FEV_1 (% predicted)	65.88 ± 26.01	70.19 ± 23.01	*52.16 ± 13.15*	66.08 ± 22.98	73.11 ± 17.99	**86.51 ± 25.24**
FVC (% predicted)	87.36 ± 20.23	92.41 ± 21.33	*77.16 ± 15.29*	88.64 ± 20.68	94.73 ± 21.41	**103.12 ± 24.49**
Atopy (% positive)	53.84%	*47.37%*	81.82%	54.84%	50.00%	**88.89%**
eNO (ppb)	37.48 ± 39.12	**48.77 ± 36.83**	37.63 ± 27.47	44.43 ± 35.75	42.11 ± 45.76	44.58 ± 43.17
Sputum eosinophils (% of inflammatory cell count)	18.74 ± 20.21	**23.08 ± 23.77**	17.03 ± 20.06	21.1 ± 22.26	7.74 ± 13.86	11.3 ± 17.85
Sputum neutrophils (% of inflammatory cell count)	63.88 ± 27.18	42.27 ± 23.32	46.1 ± 22.07	47.48 ± 25.09	49.4 ± 22.81	*40.69 ± 23.37*
Sputum macrophages (% of inflammatory cell count)	16.63 ± 21.58	33.09 ± 24.1	34.68 ± 16.89	29.92 ± 23.21	41.45 ± 25.23	47.37 ± 21.63

Highest variable values are shown in boldface, and lowest values are shown in italics.
AQLQ, Asthma Quality of Life Questionnaire; *FVC*, forced vital capacity; *ICU*, intensive care unit.

and myeloperoxidase, levels of all of which were increased. Levels of annexin A1 and A3, coronin 1A, and the chloride intracellular channel protein 1 were also increased. S100A9 is highly expressed in neutrophils, activated monocytes, and differentiated macrophages. It has several functions in cellular inflammation, responding to intracellular Ca^{2+}. Extracellular S100 proteins act as damage-associated molecular pattern proteins, initiating proinflammatory immune responses. Annexin A1, a member of a large superfamily of glucocorticoid-regulated calcium- and phospholipid-binding proteins, modulates neutrophil homeostasis and is an anti-inflammatory protein in innate immunity, modulating activation of several types of cells, including neutrophils. Annexin A1 inhibits nuclear factor κB (NF-κB) and blocks eicosanoid production by suppressing phospholipase A_2. Myeloperoxidase catalyzes the formation of hypohalous acids that have antimicrobial properties. Neutrophil gelatinase-associated lipocalin scavenges bacterial siderophores, thus depriving bacteria of iron. It is localized in azurophils and colocalizes with myeloperoxidase.[17] Azurocidin is a multifunctional inflammatory mediator with antimicrobial properties that binds endotoxin and is chemotactic for monocytes and macrophages.[18] Sputum cell gene expression in the neutrophilic phenotype was predicted to result from higher activity of the T1 cytokines IL-2 and IFN-α (see Fig E6) and IL-1β, IL-17, IFN-γ and IL-8. S100A9 protein drives neutrophilic inflammation in asthmatic patients, possibly by inducing IL-1β, IL-17, and IFN-γ. In response to inflammatory stimuli, recruited neutrophils release granular proteins, histones and chromatin DNA. These neutrophil extracellular traps amplify the efficacy of antimicrobial substances from neutrophils by maintaining a high local concentration to degrade pathogens before engulfment. Among the eosinophilic subphenotypes, the galectin-3–binding protein LG3BP was greatest in subphenotype 3 (Table III).

This study has also identified some important features outside the 2 main (eosinophilic and neutrophilic) phenotypes. IL-13 and mitogen-activated protein kinase 1–mediated gene expression signatures were identified as increased relative to the healthy state in the highly atopic phenotype (see Fig E6, *C* and *E*), which is suggestive of a T2 phenotype in this milder form of asthma characterized by lower sputum granulocyte counts. Clusterin, one of the biomarker proteins predictive of this phenotype (Fig 3 and see Fig E4, *C*), modulates NF-κB transcriptional activity[19]; however, NF-κB–mediated gene expression was not upregulated in this study (see Fig E6, *L*). The IL-1 receptor antagonist gene (IL1RN), a potent anti-inflammatory cytokine, was also activated in the highly atopic phenotype (see Fig E6, *B*). The lack of eosinophilic inflammation in this phenotype might be also in part caused by lower expression of the predictive biomarker protein plastin-2 (Fig 3, *B*), which mediates priming of eosinophils.[20] Similarly, lower expression of cofilin-1 and β-actin, proteins that involved in eosinophil priming, was predictive of classification of this highly atopic phenotype.

Use of TDA enabled an appreciation of the features of the spectrum of subphenotypes by examining the molecular and clinical characteristics across the TDA network. The order in which the clusters are shown in Table E9 (approximate left to right order) and Fig 5 (approximate circular order) should not be seen as absolute because there is some overlap between clusters and TDA networks do not have defined coordinates. Nevertheless, visualization of the broad trends is helpful to explore the relative contribution of mechanisms across the whole network, something that is not possible with other methods, such as hierarchical clustering. For example, the type I interferon IFN-α and TNF and KDM5B (JARID1B) demethylase were all identified as predicted upstream regulators of gene expression and seen as being increasingly activated from left to right in the TDA network, suggesting that the more severe granulocytic phenotypes are associated with viral and/or bacterial infections. Gene expression on the left side of the TDA network (subphenotypes 5, 6, and 4) was predicted by collagen type XVIII α1 (COL18A1), which has been shown to be associated with atopy.[21] Furthermore, the shape of the TDA network revealed a broad gap between

Highly atopic				Neutrophilic			All asthma	Healthy
6	5, 6	7	8	9	10	8, 9, 10		
32	50	*9*	27	19	11	59	200	40
16.0%	25.0%	*4.5%*	13.5%	9.50%	5.50%	29.5%		
48.87 ±15.2	48.74 ±15.42	55.44 ±8.86	*47.62 ±15.31*	52.66 ±12.19	52.81 ±13.49	50.38 ±13.94	51.34 ±13.64	37.20 ±13.11
4.9 ± 9.64	3.78 ± 8.19	**11.66 ± 17.24**	9.11 ± 15.32	6.64± 9.05	1.88 ± 3.96	5.86 ± 11.83	5.86 ± 12.58	0.28 ± 1.09
1.51 ± 1.14	1.38 ± 1.09	1.66 ± 1.42	1.95 ± 1.25	**2.3 ± 1.48**	1.49 ± 1.25	1.99 ± 1.34	1.76 ± 1.30	0.01 ± 0.06
1.82 ± 1.19	1.65 ± 1.17	2.03 ± 1.48	2.24 ± 1.28	**2.78 ± 1.66**	1.66 ± 1.23	2.32 ± 1.45	2.06 ± 1.39	0.01 ± 0.06
4.02 ± 2.25	4.37 ± 2.14	3.87 ± 2.1	4.55 ± 1.51	3.89 ± 1.88	4.33 ± 1.77	4.28 ± 1.69	4.31 ± 1.90	6.95 ± 0.08
18.75%	20.00%	11.11%	22.22%	21.05%	**63.64%**	28.81%	17.00%	0.00%
28.13%	26.00%	*22.22%*	33.33%	**57.89%**	45.45%	42.37%	33.00%	0.00%
39.23 ± 17.57	40.12 ± 24.08	*31.81 ± 24.41*	45.72 ± 16.38	49.69 ± 11.15	39.51 ± 21.89	40.94 ± 20.82	43.36 ± 24.98	38.85 ± 19.61
72.97 ± 21.31	77.84 ± 23.37	67.73 ± 24.01	69.42 ± 18.76	62.36 ± 25.83	69.17 ± 18.22	66.00 ± 21.52	69.81 ± 22.61	101.89 ± 12.72
92.18 ± 18.52	96.12 ± 21.15	89.05 ± 14.63	95.26 ± 19.04	86.37 ± 22.28	90.99 ± 15.56	90.69 ± 19.78	91.77 ± 20.47	108.71 ± 12.91
71.88%	78.00%	55.56%	77.78%	21.05%	72.73%	67.80%	63.86%	32.50%
18.67 ± 11.5	28 ± 29.54	27 ± 20.33	25.87 ± 13.65	29.72 ± 30.68	31.09 ± 26.89	27.43 ± 22.48	34.36 ± 32.4	19.56 ± 15.00
6.05 ± 13.06	7.94 ± 14.89	2.68 ± 4.36	*1.57 ± 1.49*	2.48 ± 4.55	6.26 ± 9.58	2.77 ± 5.19	10.21 ± 17.06	0.28 ± 0.53
45.79 ± 19.85	43.96 ± 21.59	46.22 ± 25.57	63.51 ± 19.87	**82.03 ± 23.71**	72.58 ± 18.26	71.79 ± 22.32	53.86 ± 25.69	38.52 ± 24.05
46.58 ± 21.48	46.86 ± 22.12	**50.12 ± 25.75**	33.38 ± 19.29	*14.76 ± 23.3*	20.45 ± 15.61	24.34 ± 21.68	34.64 ± 24.37	59.6 ± 24.36

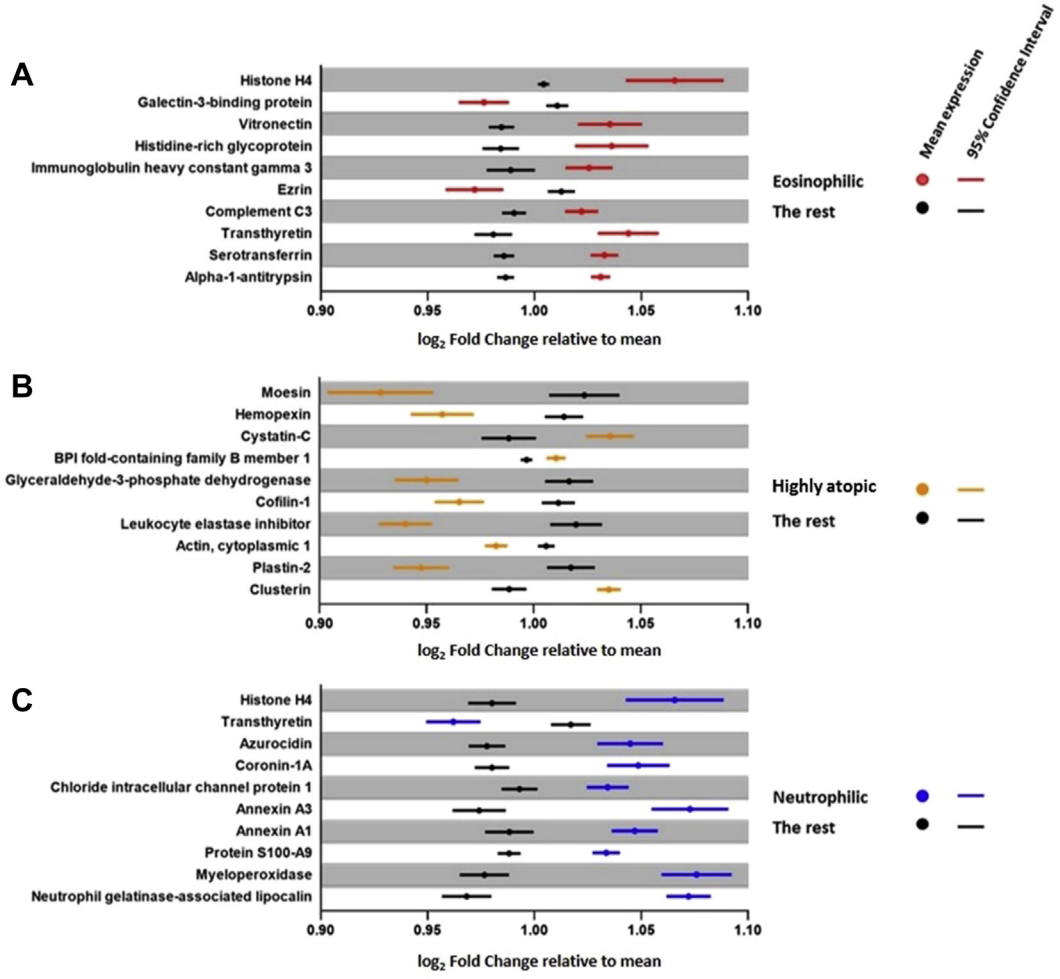

FIG 3. Sputum proteins shown by using logistic regression and ROC analysis to be most predictive of the eosinophilic **(A)**, highly atopic **(B)**, and neutrophilic **(C)** phenotypes. Expression is normalized to mean expression in all asthmatic participants samples (set to 1).

TABLE III. Sputum protein differences between subphenotypes within the eosinophilic, highly atopic, and neutrophilic asthma phenotypes identified by means of logistic regression

Phenotype	Subphenotype comparison	Protein ID	Protein name	*P* value, *t* test	Fold change (log_2)
Eosinophilic	1 vs 2 and 3	NGAL_HUMAN	Neutrophil gelatinase-associated lipocalin (NGAL)	8.00E-10	0.1
		G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	2.00E-09	0.12
	2 vs 1 and 3	HV320_HUMAN	Immunoglobulin heavy variable 3-20	2.00E-03	0.07
		TCO1_HUMAN	Transcobalamin-1 (TC-1)	3.00E-03	0.06
		MYH13_HUMAN	Myosin-13 (myosin heavy chain 13)	7.00E-03	0.11
		LDHA_HUMAN	L-lactate dehydrogenase A chain (LDH-A)	1.00E-02	−0.06
		TPIS_HUMAN	Triosephosphate isomerase (TIM)	1.00E-02	−0.06
		MYH7_HUMAN	Myosin-7 (myosin heavy chain 7)	2.00E-02	0.12
		CFAB_HUMAN	Complement factor B	3.00E-02	0.02
		AL3B1_HUMAN	Aldehyde dehydrogenase family 3 member B1	3.00E-02	0.05
		ILEU_HUMAN	Leukocyte elastase inhibitor (LEI) (monocyte/neutrophil elastase inhibitor)	4.00E-02	−0.05
	3 vs 1 and 2	CLUS_HUMAN	Clusterin (complement cytolysis inhibitor)	2.00E-04	0.05
		LG3BP_HUMAN	Galectin-3-binding protein	9.00E-04	0.04
		PROL4_HUMAN	Proline-rich protein 4	2.00E-03	0.07
Highly atopic	5 vs 6	LYSC_HUMAN	Lysozyme C	5.00E-03	−0.01
Neutrophilic	8 vs 9 and 10	TKT_HUMAN	Transketolase (TK)	4.00E-11	−0.07
		CATA_HUMAN	Catalase	3.00E-10	-0.1
		PEDF_HUMAN	Pigment epithelium-derived factor (PEDF)	7.00E-08	0.08
		LG3BP_HUMAN	Galectin-3-binding protein (basement membrane autoantigen p105)	2.00E-07	0.05
	9 vs 8 and 10	BPIB1_HUMAN	BPI fold-containing family B member 1	5.00E-04	−0.01
		MUC1_HUMAN	Mucin-1 (MUC-1)	3.00E-03	−0.04
		CFAB_HUMAN	Complement factor B (C3/C5 convertase [glycine-rich beta glycoprotein])	4.00E-03	0.04
		ALDOA_HUMAN	Fructose-bisphosphate aldolase A (lung cancer antigen NY-LU-1)	4.00E-03	0.04
		ZG16B_HUMAN	Zymogen granule protein 16 homolog B	6.00E-03	−0.06
	10 vs 8 and 9	IGHG1_HUMAN	Immunoglobulin heavy constant gamma 1 (Ig gamma-1 chain C region)	2.00E-06	0.03
		HEMO_HUMAN	Hemopexin (Beta-1B-glycoprotein)	4.00E-05	0.06
		A1AT_HUMAN	Alpha-1-antitrypsin	3.00E-04	0.03
		FIBG_HUMAN	Fibrinogen gamma chain	7.00E-04	0.09
		TKT_HUMAN	Transketolase (TK)	1.00E-03	0.05
		TALDO_HUMAN	Transaldolase	2.00E-03	0.06

eosinophilic and neutrophilic asthma proteotypes (Fig 1). However, as indicated by the links between eosinophilic subphenotype 1 and neutrophilic subphenotype 10 (see lines joining these in Fig 1), the proteomes of the eosinophilic and neutrophilic subphenotypes can have some associations. In contrast, the absence of links between subphenotypes 4 and 10 indicated large proteomic differences. The highly atopic phenotypes (5 and 6) had the lowest asthma symptoms (ACQ score) and the best lung function (FEV_1, 78% of predicted value), lower symptom severity, and hence best quality of life compared with the highly granulocytic phenotypes. At the opposite right end of the network were subphenotypes 1 and 10, in which asthma symptom (ACQ) scores were most severe, suggesting that the asthma spectrum progresses in severity from the left to right ends of the network, worsening either through the neutrophilic or eosinophilic pathways. There was increasing IL-2–mediated and decreasing IL-13 gene expression from left to right across the TDA network, suggesting a shift from T2 to T1 mechanisms with increasing severity (Fig 5). Additionally, there was a trend across the TDA structure (Fig E6, *I*, and see Table E9) of gene expression associated with the upstream regulator Brahma-related gene-1 (also known as SMARCA4), a chromatin-remodeling factor known to inhibit expression of CD44[22] and E-cadherin,[23] drivers of the T2 phenotype.[24]

Unsurprising for a study of severe asthma, OCS use was high, with mean percentages of patients requiring OCSs for disease control ranging from 27% in eosinophilic subphenotype 3 to 58% in neutrophilic subphenotype 9. Not surprisingly, OCS use was highest in patients with neutrophilic subphenotypes, possibly because of the proneutrophilic effects of corticosteroids on neutrophil numbers, although the higher rates of intensive care unit admission in these patients (Fig 2, *L*) suggest particularly severe pathogenetic mechanisms that result in the most severe forms of exacerbation. We speculate that in the atopic, predominantly paucileukocytic subphenotypes corticosteroids were effective at reducing eosinophilic inflammation, whereas the persistence of

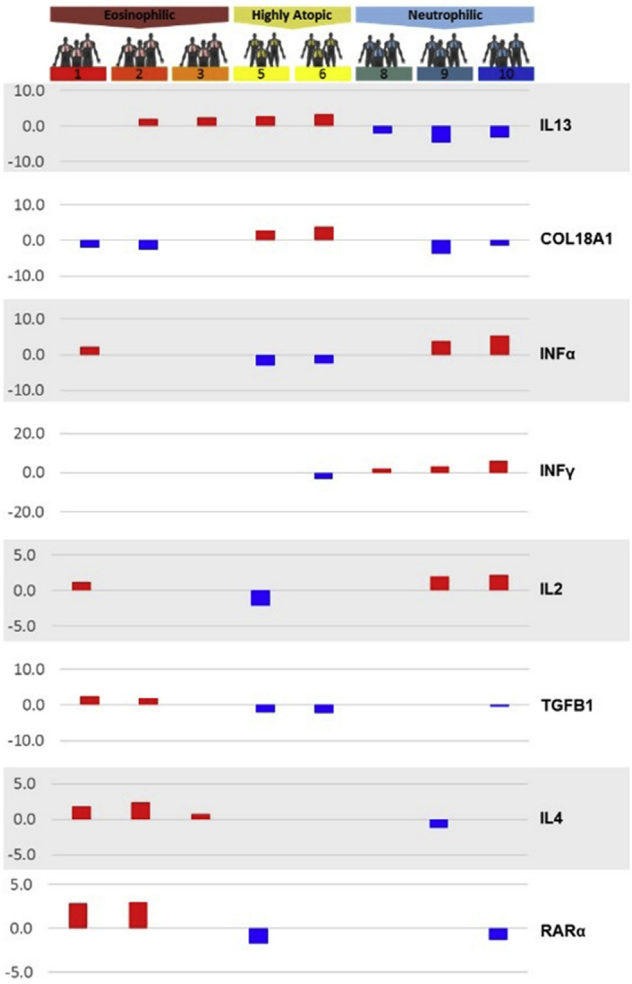

FIG 4. Selected top upstream regulators of gene expression across the subphenotypes of eosinophilic, highly atopic, and neutrophilic asthma phenotypes. The sequence of subphenotypes is shown in an approximate circular order, beginning with the highly eosinophilic subphenotypes (clusters) 1, 2, and 3; moving through the highly allergic subphenotypes 5 and 6; and ending with the highly neutrophilic subphenotypes. *RARα*, Retinoic acid receptor α.

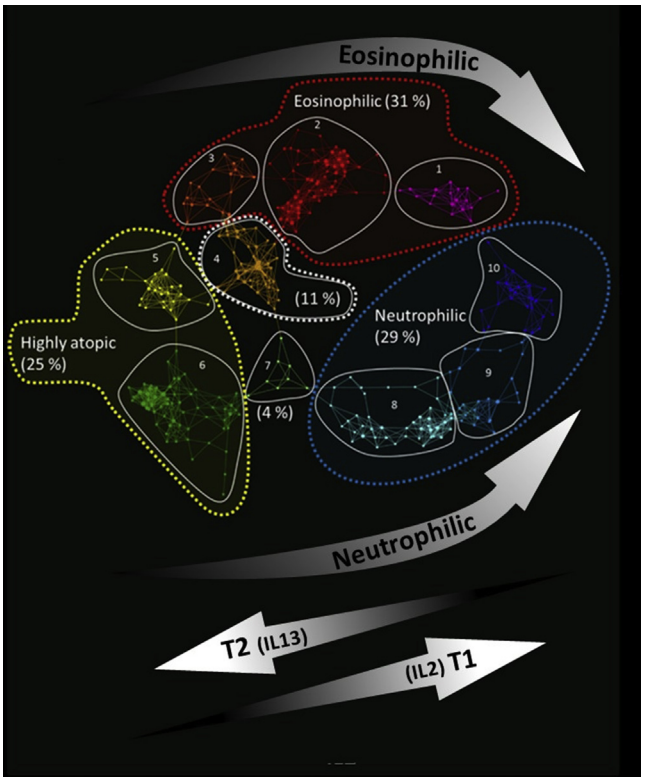

FIG 5. Pattern across the TDA structure of activation of IL-13 and IL-2, upstream regulators of gene expression, are representative of T2 and T1, respectively. Also shown are *arrows* indicating increasing neutrophil and eosinophil cell counts.

eosinophilic inflammation in subphenotypes 1 to 3 points to at least partial insensitivity to corticosteroids. Full elucidation of the mechanisms to explain the levels of granulocytic inflammation and responses to OCSs requires appropriately designed mechanistic studies.

This study has limitations. The individual biomarkers and subphenotypes need to be validated in a separate (validation) cohort and assessed for intrasubject reproducibility in a longitudinal study. Stratification of the highly eosinophilic, neutrophilic, and atopic phenotypes into subphenotypes resulted in insufficient numbers of participants in the subphenotypes, prohibiting the extensive analysis we were able to conduct on the phenotypes.

Furthermore, like other studies involving large cohorts, we have not undertaken a stringent analysis of adherence to treatment by using methods like fraction of eNO suppression testing, which have been recently validated.[25] Such additional analyses and application of biomarkers in mechanistic studies with new asthma drugs, especially the range of biologics available and others in development, could point to further associations with exacerbations and provide why some but not all patients benefit from individual biologics.[26,27] As in all studies of severe asthma, treatments varied significantly between participants, and these variations are likely to affect the biomarker profiles. Perhaps of greatest relevance is the variable use of OCSs, even though there is hope that the majority of patients will no longer be dependent on them as maintenance treatment.

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Clinical implications: By further stratifying asthma, the sputum proteomes and molecular pathways identified in this study could explain the variability in treatment response to asthma therapies, in particular the biologics.

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